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High Prevalence of GB Virus C/Hepatitis G Virus Infection Among Homosexual Men Infected With Human Immunodeficiency Virus Type 1: Evidence for Sexual Transmission

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GB virus C/hepatitis G virus (GBV-C/HGV), a recently discovered orphan flavivirus, is distantly related to hepatitis C virus (HCV). Although both GBV-C/HGV and HCV can be transmitted by the parenteral route, their principal modes of transmission and associated risk behaviors may differ. Using reverse transcription-polymerase chain reaction, the 5′-noncoding regions of GBV-C/HGV and HCV were amplified from plasma or sera of 209 individuals infected with human immunodeficiency virus type 1 (HIV-1). As verified by Southern blot analysis, GBV-C/HGV and HCV infection were detected in 37 (17.7%) and 22 (10.5%) of 209 HIV-1-infected individuals, respectively. GBV-C/HGV infection was significantly associated with homosexual sex (P = 0.044) and was more common than HCV infection among HIV-1-infected homosexual men (P = 0.006). The prevalence of GBV-C/HGV infection was nearly equal in women infected with HIV-1 via high-risk heterosexual sex (14.0%) or injection drug use (IDU) (17.5%). By contrast, HCV infection was associated significantly with women reporting IDU when compared to women reporting high-risk heterosexual sex (P < 0.0001). Alanine aminotransferase levels were elevated in HIV-1-infected individuals who were co-infected with HCV (P = 0.009), but not with GBV-C/HGV (P = 0.9). The high prevalence of GBV-C/HGV infection in HIV-1-infected nondrug-injecting homosexual men and among women engaging in high-risk heterosexual sex is consistent with transmission by the mucosal route and with acquisition of infection by the receptive rather than insertive partner. ***J. Med. Virol. 56: 123–127, 1998.***

KEY WORDS: HGV; GBV-C; HCV; HIV; homosexual; IDU; PCR

INTRODUCTION

GB virus C or hepatitis G virus (GBV-C/HGV) and hepatitis C virus (HCV) are distantly related members of the *Flaviviridae* family, exhibiting 29% sequence homology at the amino acid level [Linnen et al., 1996; Simons et al., 1995a,b]. During the past 30 years, ~4 million people in the United States and millions more worldwide have been infected with HCV. HCV-associated chronic liver disease and hepatocellular carcinoma are predicted to emerge as important medical problems in the next two decades [Marwick, 1997]. In individuals infected with human immunodeficiency virus type 1 (HIV-1), concurrent infection with HCV, usually acquired by injection drug use (IDU), can rapidly accelerate the progression to acquired immune deficiency syndrome (AIDS) and/or liver failure [Bierhoff et al., 1997; Soto et al., 1997].

Much less information is available on the prevalence, preferred transmission routes, and disease potential of GBV-C/HGV infection in HIV-1-infected individuals. Because of the close association between GBV-C/HGV and HCV, it has been difficult to deduce the pathogenic potential of GBV-C/HGV. That is, few cases of individuals infected only with GBV-C/HGV have been studied in detail. Whereas preliminary research has indicated limited pathogenicity of GBV-C/HGV, when compared

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to HCV, in causing chronic hepatitis, the findings do not exclude a causative association between GBV-C/HGV and other disorders.

A reverse transcription-polymerase chain reaction (RT-PCR) was employed to amplify the 5′-noncoding regions of GBV-C/HGV and HCV from HIV-1-infected individuals. The data indicate that GBV-C/HGV is transmitted more efficiently than HCV by the mucosal route and that GBV-C/HGV is not a passenger virus but is transmitted independently of HCV.

MATERIALS AND METHODS

Study Population

Stored residual serum or plasma, obtained during March 1994 to March 1997 and stored under coded identifier numbers, were studied. Blood specimens were available from 209 HIV-1-infected patients, as part of their primary care and/or in the context of their participation in clinical research protocols approved by the Committee on Human Subjects of the University of Hawaii at Manoa. Demographic and pertinent clinical data, retrieved by coded identifier, were provided by the Hawaii AIDS Research Consortium. Because of the retrospective nature of this study, with samples originating from individuals on different protocols, consistent and complete data on various parameters, such as CD4 count and HIV-1 viral load, were not always available for each patient. HIV-1 infection was diagnosed by Western blot analysis and/or PCR.

Of the 209 HIV-1-infected patients (136 men, 73 women), 130 (62.2%) were Caucasians, 58 (27.8%) were Asians and Pacific Islanders and 21 (10.0%) were classified as other (four African Americans, four Native Americans, 11 nonwhite Hispanics, and two unknown). The presumed mode of acquisition of HIV-1 infection was homosexual sex in 106 (50.7%), high-risk heterosexual sex in 65 (31.1%), IDU in 27 (12.9%), and unknown in 11 (5.3%). Nearly 80% of the study population was 25–44 years old (age range, 20–60 years; mean and median age, 36 years).

Biochemical Assay for Alanine Aminotransferase

Alanine aminostransferase (ALT) was measured in serum by a three-point kinetic assay using an automated photometric analyzer (model 917, Hitachi-Boheringer Mannheim, IN). Levels were expressed in international units (normal range, 0–40 IU/L).

Extraction of RNA and Synthesis of First-Strand cDNA

Total RNA was extracted and purified from 560 μL of plasma or serum using the QIAamp Viral RNA Kit (QIAGEN, Chatsworth, CA). All samples were eluted in 75 μL of diethylpyrocarbonate-treated distilled water and were stored at −80°C until use.

First-strand cDNA was synthesized as follows: 20 μL reaction mixture containing 8 μL total RNA, 500 ng random hexamers (Promega, Madison, WI), 1.0 mM each dNTP, 10 mM dithiothreitol, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3.0 mM $MgCl_2$, and 150 U Superscript reverse transcriptase-H^- (Life Technologies GIBCO-BRL, Gaithersburg, MD), was incubated at 25°C for 10 min, then at 42°C for 50 min, and heated at 72°C for 15 min using a GeneAmp System 2400 DNA Thermal Cycler (Perkin-Elmer, Norwalk, CT). cDNA were stored at −80°C until further use.

RT-PCR Diagnosis of GBV-C/HGV and HCV Infection

cDNA generated with random primers was used for amplification of the highly conserved 5′-noncoding region of GBV-C/HGV and HCV using oligonucleotide primers derived from sequences of GBV-C/HGV strain PNF2161 (GenBank accession no. U44402) and HCV strain HCV-H/HCV-1 (GenBank accession no. D10749) [Linnen et al., 1996; Novati et al., 1992], respectively. Primers were used in a nested fashion for amplification of GBV-C/HGV (outer primers: bases 26 to 45, 5′-CCGGCACTGGGTGCAAGCCC-3′, and bases 534 to 515, 5′-AAATGCCACCCGCCCTCACC-3′; inner primers: bases 100 to 119, 5′-CGGCCAAAAGGTGGTGGATG, and bases 475 to 453, 5′-GAGCTGGGTGGCCCCATGCATTT-3′). For HCV amplification, primers were: bases 82 to 101, 5′-GCCATGGCGTTAGTATGAGT-3′, and bases 339 to 320, 5′-TGCACGGTCTACGAGACCTC-3′.

"Hot start" PCR was carried out in a reaction mixture of 20 μL containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.8 mM $MgCl_2$, 0.1 μM each primer and 0.2 mM each dNTP. Each mixture was overlaid with a bead of PCRGem 50 Ampliwax (Perkin-Elmer), heated to 80°C for 5 min, and cooled at room temperature. A reaction mixture of 30 μL containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.25 U of *Thermus aquaticus* DNA polymerase (Perkin-Elmer), and 5 μL of cDNA was then added on top of the solidified wax. Using a thermal cycler, the mixtures were initially denatured at 94°C for 2.5 min, then cycled 10 times at 94°C for 15 sec, 45°C for 30 sec, 72°C for 90 sec, followed by 35 cycles at 94°C for 15 sec, 50°C for 30 sec, and 72°C for 90 sec before storing at 4°C. For nested PCR, 5 μL of the original amplicon was cycled 45 times at 94°C for 15 sec, 55°C for 30 sec, and 72°C for 60 sec. Amplicons were size-fractionated, then visualized on 2% ethidium bromide-stained agarose gels.

GBV-C/HGV- and HCV-specific gene sequences were detected by dot-blot hybridization, using specific oligonucleotide probes labeled by a nonradioactive method (DIG/Genius™ System, Boehringer Mannheim, Indianapolis, IN): for GBV-C/HGV, bases 161 to 179, 5′-GGTAGCCACTATAGGTGGG-3′; for HCV, bases 127 to 144, 5′-TCCCGGGAGAGCCATAGT-3′. Additionally, representative GBV-C/HGV- and HCV-positive amplicons were cloned using the TA cloning system (Invitrogen, San Diego, CA), and one or more clones for each amplicon was sequenced in both directions using the Dye Primer Cycle Sequence Ready Reaction Kit on an automated sequencer (model 373A, Applied Biosystems, Foster City, CA).

TABLE I. Serum ALT Levels in 110 HIV-1-infected Individuals Based on GBV-C/HGV and HCV Status

GBV-C/HGV and HCV status[a]	No.	ALT level (IU/L)[b]			
		Minimum	Maximum	Mean	Median
GBV-C/HGV (pos)	28	13	202	44.4	30.5
GBV-C/HGV (neg)	82	7	219	43.2	32.5
HCV (pos)	15	19	219	71.2*	46.0
HCV (neg)	95	7	188	39.1	30.0
GBV-C/HGV (pos) HCV (neg)	25	13	111	39.3	30.0
GBV-C/HGV (neg) HCV (neg)	70	7	188	39.6	30.0
GBV-C/HGV (neg) HCV (pos)	12	19	219	67.3*	47.0
GBV-C/HGV (pos) HCV (pos)	3	27	202	86.7	31.0

[a]pos = positive; neg = negative.
[b]ALT normal range, 0–40 IU/L.
*P = 0.009, Kruskal-Wallis H test.

Sensitivity and Specificity of GBV-C/HGV and HCV PCR

To ascertain the sensitivity and specificity of the GBV-C/HGV and HCV PCR, RNA and cDNA samples were selected randomly and diluted serially fourfold. Briefly, 8 μL of total RNA were diluted fourfold from 1:4 to 1:256, then reverse transcribed using the above protocol. Similarly, 5 μL of cDNA was diluted serially fourfold from 1:4 to 1:256. Undiluted and serially diluted cDNA and cDNA generated from serially diluted RNA were subjected to GBV-C/HGV and HCV PCR, as described above. Amplicons were then electrophoresed on 2% agarose gels. For specificity, all GBV-C/HGV and HCV amplicons were detected using GBV-C/HGV- and HCV-specific oligonucleotide probes. GBV-C/HGV and HCV amplicons, amplified from serially diluted RNA and cDNA, were detected in dilutions from 1:4 to 1:64 by ethidium-bromide fluorescence and chemiluminescence (data not shown).

Data Analysis

Epidemiological and clinical data were analyzed using Epi Info Version 6.0 [Dean et al., 1994] and SPSS Version 7.0 (SPSS, Chicago, IL). Student's *t*-test and one-way analysis of variance were used to test differences in means of continuous variables between independent groups. When the variances of samples differed significantly or when the samples were not normally distributed, the nonparametric Mann-Whitney *U* test (two means) or Kruskal-Wallis H test (three or more means) were used to compare differences in means. Associations between dichotomous epidemiological and clinical variables in 2 × 2 tables were < 5. Multiple linear regression was used to analyze relationships between independent and dependent variables.

RESULTS

ALT Levels

Serum ALT levels were available for analysis from 110 (52.6%) participants. No differences were apparent with respect to age, gender, and ethnicity of participants with and without serum ALT measurements. A significant elevation in ALT was found in HCV RNA-positive HIV-1-infected individuals ($P = 0.009$) but not in those co-infected with GBV-C/HGV ($P = 0.9$) (Table I). Given similar gender, ethnicity, and age, the conversion from HCV RNA positivity to negativity contributed to a significant decrease in ALT levels ($P < 0.001$). In three HIV-1-infected persons co-infected with both GBV-C/HGV and HCV, serum ALT levels were 27, 31, and 202 IU/L.

Analyses of GBV-C/HGV and HCV Infection

As verified by dot-blot hybridization and DNA sequencing, GBV-C/HGV infection was detected in 37 individuals (17.7%), whereas HCV infection was found in 22 of the 209 (10.5%) HIV-1-infected study participants. GBV-C/HGV infection was more common than HCV infection among HIV-1-infected homosexual men ($P = 0.006$) (Table II). GBV-C/HGV positivity was associated ($P = 0.044$) with homosexual sex (24.5%) rather than high-risk heterosexual sex (10.8%) or IDU (14.8%). Neither GBV-C/HGV nor HCV positivity were associated significantly with high-risk heterosexual sex. By contrast, HCV infection was associated significantly with IDU ($P < 0.0001$), even after stratifying for gender, 5-year age group and ethnicity ($P = 0.002$).

Eight of 17 (47.1%) HIV-1-infected women reporting IDU were infected with HCV, in comparison with 2 of 50 (4.0%) women reporting high-risk heterosexual sex ($P < 0.0001$). By contrast, the prevalence of GBV-C/HGV infection was nearly equal among women infected with HIV-1 by IDU (17.6%) or high-risk heterosexual sex (14.0%)($P = 0.7$), suggesting a more prominent role for sexual transmission of GBV-C/HGV. Of the 10 HIV-1-infected men reporting IDU, one (10.0%) and two (20.0%) were infected with GBV-C/HGV and HCV, respectively. None of the 15 HIV-1-infected men reporting high-risk heterosexual sex and none of the 11 individuals whose HIV-1 risk factors were unknown were infected with GBV-C/HGV or HCV. Dual infection with GBV-C/HGV and HCV was found in only three (1.4%) individuals (two IDU and one homosexual man).

A follow-up specimen was available from 18 of the 37 (48.6%) GBV-C/HGV RNA-positive individuals. All but two (both homosexual men) of these 18 individuals remained GBV-C/HGV RNA positive 2–30 months later (median, 16.5 months).

TABLE II. Gender-specific Prevalences of GBV-C/HGV and HCV Infection in 209 HIV-1-infected Individuals*

Presumed mode of HIV-1 infection	GBV-C/HGV			HCV		
	Men	Women	Total	Men	Women	Total
Homosexual sex	26/106	0	26/106 (24.5)	10/106	0	10/106 (9.4)
High-risk heterosexual sex	0/15	7/50	7/65 (10.8)	0/15	2/50	2/65 (3.1)
Injection drug use	1/10	3/17	4/27 (14.8)	2/10	8/17	10/27 (37.0)
Unknown	0/5	0/6	0/11	0/5	0/6	0/11

*Prevalences expressed as percentages in parenthesis.

DISCUSSION

Since the discovery of GBV-C/HGV, attempts have been made to clarify its principal mode of transmission [Moaven et al., 1996; Chen et al., 1997; Wu et al., 1997], as well as its disease potential. In this study, we addressed these issues among HIV-1-infected individuals. Since serum antibodies against GBV-C/HGV were not assessed, prevalences of GBV-C/HGV infection based on RT-PCR must be viewed as minimal rates. GBV-C/HGV prevalences of 24.5%, 10.8%, and 14.8%, respectively, were found among individuals infected with HIV-1 via homosexual sex, high-risk heterosexual sex, and IDU. These data contrast with a recent report showing GBV-C/HGV prevalences of 0%, 25.0%, and 42.6% in these respective groups [Fiordalisi et al., 1997]. The primary difference between these studies was the number of individuals in each group. Our study population consisted primarily of homosexuals (106 or 50.7%) and heterosexuals (65 or 31.1%) and few IDU (27 or 12.9%), whereas homosexual men (11 or 11.8%) were few and heterosexual partners of IDU (28 or 30.1%) and IDU (54 or 58.1%) were predominant in the previous study [Fiordalisi et al., 1997].

The near absence of GBV-C/HGV infection among heterosexual men (1/25, 4.0%) and the comparatively higher prevalences of GBV-C/HGV infection among heterosexual women (10/65, 15.3%) suggest that, as in HIV-1 infection, the receptive partner is at a higher risk for acquiring GBV-C/HGV. Sexual transmission of GBV-C/HGV is also supported by data of Rubio et al. [1997], who reported an GBV-C/HGV prevalence of 21.7% among heterosexual partners of 150 index cases. Moreover, GBV-C/HGV prevalences of 13.9% and 13.4% were found among female prostitutes and homosexual men, respectively. Similarly, Stark et al. [1996] found an GBV-C/HGV prevalence of 10.9% among non-drug-injecting homosexual and bisexual men who had engaged in unprotected insertive or receptive anal intercourse. Finally, Kao et al. [1997] demonstrated a positive correlation between GBV-C/HGV infection in prostitutes and the number of years of prostitution. Although previously reported prevalences of GBV-C/HGV infection among homosexual men are somewhat lower than that in our study, they indicate collectively that GBV-C/HGV, unlike HCV, is frequently transmitted by the sexual route.

Of 49 HCV-associated chronic liver disease patients with a history of IDU, 12 (24.4%) were positive for GBV-C/HGV, compared to nine of 128 (7.0%) patients with HCV-associated liver disease with no history of IDU [Aikawa et al., 1996]. By contrast, our data on HIV-1-infected IDU showed prevalences of 37.0% and 14.8% for HCV and GBV-C/HGV, respectively, and rather than a co-infection rate of 24.4%, a 1.4% prevalence of dual infection with GBV-C/HGV and HCV was found. Similarly, GBV-C/HGV infection has been reported in one of 17 (5.9%) patients with HCV-associated chronic liver disease and tattoos but no history of IDU, which suggests that such procedures may not be an efficient mode of GBV-C/HGV transmission [Aikawa et al., 1996]. These relatively low prevalencs of GBV-C/HGV and HCV co-infection argue against GBV-C/HGV being merely a passenger virus.

In this study, as in other published reports, no association was found between liver dysfunction (as determined by serum ALT measurements) and GBV-C/HGV persistence, irrespective of the associated risk behaviors [Thomas et al., 1997]. Instead, elevated levels of ALT were associated significantly with HCV infection, and gender and ethnicity did not play any role in the levels of ALT. Additionally, the conversion from HCV RNA positivity to negativity contributed to a significant decrease in ALT levels.

To what extent GBV-C/HGV persistence leads to chronic liver disease is unclear. In a study examining GBV-C/HGV persistence and disease, GBV-C/HGV infection was detected in nine of 29 short-term IDU, five of whom (55.5%) cleared the GBV-C/HGV over 5.8 years [Thomas et al., 1997]. In our study, two of 18 (11.1%) HIV-1-infected individuals no longer had detectable plasma GBV-C/HGV RNA 4 and 27 months later. The lower percentage of virus clearance in our study participants may be attributed to the shorter duration of follow-up and/or to HIV-1-induced immunosuppression. Unfortunately, the study design did not permit us to determine whether GBV-C/HGV infection predated acquisition of HIV-1. Collectively, the above data indicate that GBV-C/HGV infection is not associated with elevated ALT and that it is independent of HCV infection. Whether or not persistent GBV-C/HGV infection affects the rate of progression to AIDS remains unknown.

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